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APPENDIX 3

Characterization of two distinct P2Y receptors in human tracheal gland cells

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Human submucosal tracheal glands are now believed to play a major role in the physiopathology of cystic fibrosis, a genetic disease in which ATP is used as a therapeutic agent. However, actions of ATP on tracheal gland cells are poorly known. ATP-binding characteristics, and ATP-induced formation of cAMP were investigated in a cell line (MM39) of human tracheal gland cells. The binding of a radiolabelled non-hydrolysable analogue of ATP Adenosine-5′-[^{35}S]thiotriphosphate: [^{35}S]ATP[γS] was rapid (within 30 min at 4 °C), stable and reversible. Scatchard analysis revealed two classes of [^{35}S]ATP[γS]-binding sites. Low-affinity binding sites had a K_d1 of 20 ± 5 μM (B_{max} = 150 nmol/10^6 cells) and the high-affinity binding sites had a K_d2 of 2.5 ± 0.2 μM (B_{max} = 52 nmol/10^6 cells). Competition experiments showed competition with ATP, ADP and 2-methylthio-ATP but no competition with UTP, AMP and adenosine. UTP stimulates protein secretion as well as it induced $[Ca^{2+}]_i$ mobilization but did not affect the intracellular cAMP levels. ATP also caused induced $[Ca^{2+}]_i$ mobilization and protein secretion but also caused an increase in cyclicAMP content of the cells, reaching a maximum after 1 min. ATP-induced cAMP formation was concentration dependent and inhibited by the P2-antagonist suramin. Reverse-transcription-PCR amplification revealed the presence of the transcripts of both the P2Y2 and the UTP-specific P2Y4 receptors. In conclusion, P2Y2 receptors, UTP-P2Y4 receptors and unidentified ATP-specific receptors seem to be present in MM39 cells which appear to be coupled differently to intracellular second-messenger systems.

Keywords: airway; purinoceptor; pyrimidinoceptor; binding; suramin.

Cystic fibrosis (CF) is a fatal hereditary disease, the most serious syndrome being mucus overabundance and persistent infection of the airways. This disease, which affects all exocrine glands is now recognized as the result of mutations in a gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) that leads to a defect in cAMP-stimulated chloride transport [1]. Subsequently, mucus may become altered in its rheological properties and bacterial adhesivity, leading to inflammation and then mucus hypersecretion.

ATP is a potent secretagogue which binds to nucleotide P2-receptors resulting in intracellular calcium mobilization through phospholipase C (PLC) and also in PLD, PLA2 and mitogen-activated protein kinase (MAPK) activation. Several studies have shown that respiratory epithelia contain a chloride channel, distinct from CFTR, which can be activated by an increase in intracellular calcium and which is not defective in CF [2]. Therefore, nucleotides such as ATP and UTP have been proposed as therapeutic agents to bypass the altered function of CF and to restore chloride transport in CF patients [3].

P2 receptors were first classified pharmacologically, based on potency order of agonists [4]. They were subdivided in six classes. P2x are receptor-operated cationic channels selectively recognized by α, β-methylene ATP. P2Y receptors were first characterized as phospholipase-C-coupled heptahelicoidal receptors recognized by 2-methylthio-ATP (2-MeSATP). P2t receptors are ADP-specific receptors of platelets. P2z receptors constitute non-selective membrane pores mediating the permeabilizing effects of ATP4- and are specifically recognized by 3′-*O*-(benzoyl)-benzoyl ATP. P2D receptors are recognized by dinucleotide polyphosphates. P2u receptors are also phospholipase-C-coupled heptahelicoidal receptors but are recognized by ATP and UTP. Since the cloning of the P2u, P2Y, and P2x receptors, a complete reorganization of these receptors has been proposed in which P2Y and P2u receptors were respectively called P2Y1 and P2Y2 receptors [5]. Other P2Y receptors are now identified, the P2Y3 receptor [6] found in chick but not yet in human, the P2Y4 receptor specific for UTP [7, 8], and now the P2Y5, P2Y6 and P2Y7 receptors (for review, see [5]). Recently, the P2Y5 and the P2Y7 receptors first described as ATP-specific receptors [9, 10] were clearly demonstrated as not able to transduce any second-messenger signals [11, 12] and consequently are no longer considered as purinoceptors.

Recently, by using immunohistochemical and *in situ* hybridization techniques, evidence was given of a high expression of CFTR in the serous component of the tracheal glands while it is almost undetectable in the other cells of the human bronchus [13]. Consequently, human tracheal gland serous (HTGS) cells may represent an important target for the therapy of cystic fibrosis [14].

We developed techniques to culture HTGS cells [15, 16]. As *in vivo*, cultured HTGS cells become highly polarized, secrete the secretory leucocyte proteinase inhibitor (SLPI), lactoferin, lysozyme, and high molecular-mass macromolecules, and are re-

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Abbreviations. ATP[γS], adenosine-5′-thiotriphosphate; 2-MeSATP, 2-methylthio-ATP; RT, reverse transcription; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HTGS, human tracheal gland serous; SLPI, secretory leucocyte proteinase inhibitor; PLC, phospholipase C; LDH, lactate dehydrogenase; PLD, phospholipase D; PLA2, phospholipase A2; MAPK, mitogen-activated protein kinase; SV40, simian virus 40.

sponsive to adrenergic, cholinergic and peptidergic agents [17]. At present, only one report has generated interest in the purinergic regulatory mechanism in cultured HTGS cell secretion [18]. We have demonstrated that both ATP and UTP generated an intracellular-calcium mobilization mainly through the PLC-Ca^{2+} pathway and that they stimulated the secretion of both SLPI and high-molecular-mass macromolecules. The receptor for these ATP actions was preliminary characterized as a P2u purinoceptor based on the potency order of agonists.

Recently, we established and characterized a simian-virus-40 (SV40)-transformed cell line of HTGS cells, the MM39 cell line [19] which has retained the secretory characteristics of the original cells. In this study, we have further examined the properties of this P2 receptor by ligand-binding experiments on MM39 cells in order to provide a molecular basis for subsequent studies concerning the mechanisms of purinergic regulation of secretion by HTGS cells. We herein demonstrate the presence of both unusual purinoceptor coupled to generation of cAMP and a pyrimidinoceptor not coupled to generation of cAMP.

MATERIALS AND METHODS

Cell culture. Culture of the SV40-transformed human tracheal gland serous cell line (MM39 cell line) was performed as previously described [19]. Cells were cultured in a Dulbecco's modified Eagle's medium (DMEM/F12) mixture supplemented with 1% of the serum substitute Ultroser G (Biosepra), 0.22 g/l sodium pyruvate and 8 g/l glucose. Epinephrine (2.5 μM from a 2.5 mM stock solution made in HCl M/1000 and stored at −80°C) was routinely added to the cell culture medium in order to provide optimal growth and differentiation [17]. Cells were passaged using 0.025% trypsin (GIBCO) and 0.02% EDTA. Type I collagen-coated, Falcon disposable tissue culture flasks and 24-well plates were used. In these culture conditions, MM39 cells were reported to have conserved the physiological characteristics of the genuine cells such as the presence of cytokeratin, the expression of CFTR, and a purinergic regulated secretion of SLPI [19]. At the end of all incubations with pharmacological agents, cell viability was determined by the measurement of lactate dehydrogenase (LDH) released in the incubation medium. Samples of supernatants (30 μl) were assayed for LDH activity using the Sigma lactate dehydrogenase kit, which is a spectrophotometric assay using pyruvate and NADH as substrates.

Measurements of [^{35}S]ATP[γS] binding. For saturation experiments, confluent cells in 24-well plates were washed twice for 1 h with DMEM/F12 medium. We then added, for 1 h at 4°C, increasing concentrations (0.001 μM to 100 μM) of a mixture of Adenosine-5′-[γ-thio]triphosphate (ATP[γS]; 0.1 Ci/mmol; Amersham), in a constant volume of HBS: 10 mM Hepes, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaSO_4$ and 1 g/l glucose (300 μl/well), containing 1 mM sodium thiophosphite (which reduces non-specific binding of ATP[γS]; [20]). Supernatants were discarded and cells were rinsed three times in HBS buffer and bound radioactivity was removed after lysis of the cells with an SDS-lysis buffer (300 μl/well of HBS containing 0.1% SDS) and analysed by liquid-scintillation counting using 10 ml/well of ACS (Amersham). Non-specific binding experiments were carried out under the same conditions but with an excess of ATP (2.5 mM). B_{max} and K_dS were determined by using the non-linear LIGAND program [21].

For kinetic experiments, [^{35}S]ATP[γS] (0.1 Ci/mmol) was added to the cells and the bound radioactivity was determined after different times of incubation. In dissociation experiments, ATP[γS] was equilibrated with the cells for 60 min and dissociation was initiated by the addition of 2.5 mM unlabelled ATP.

For competition experiments, cells were incubated in the presence of 0.1 μM [^{35}S]ATP[γS] for 1 h exposure in the absence or the presence of the following analogues: UTP, ATP, ADP and 2-MeSATP, AMP and adenosine (1 nM to 1 mM). Non-specific binding was determined in the presence of 2.5 mM ATP.

2-MeSATP was from RBI. All other chemicals and drugs were from Sigma.

Reverse-transcription (RT)-PCR amplification. Total RNA was purified from cellular pellets of MM39 cells (approximately 10^7 cells) following the technique of Chomczynski et al. [22]. In detecting the expression of P2Y2 [23] and of P2Y4 [8] mRNA transcripts, PCR amplification of mRNA (after conversion to cDNA) was performed using the Gene AMP RNA PCR kit (Perking Elmer/Cetus). Specific amplifications were performed using as primers 5′-CTTCAACGAGGACTTCAAGTACGTGC-3′ (nucleotide positions: 323−348 of the P2Y2 gene) and (5′-CATGTTGATGGCGTTGAGGGTGTGG-3′ (nucleotide positions 1079−1103 of the P2Y2 gene); and 5′-ATCCTGCCACCCTCACTTCTCC-3′ (nucleotide positions 137−159 of the P2Y4 gene) and 5′-AGGCGAGAAGACGACTGTGC-3′ (nucleotide positions 822−902 of the P2Y4 gene). 35 cycles of amplification were used with a cycle pattern as follows: denaturation 60 s at 94°C, primer annealing for 60 s at 56°C (P2Y2) or 55°C (P2Y4) and extension for 150 s at 72°C. An aliquot of the final amplification solution was analysed after ethidium bromide staining of a 2% agarose gel to assess the size of the amplified fragments. Another aliquot was removed and digested by the restriction enzyme *PstI* which cuts once the two PCR products, giving two fragments of 500 bp and 281 bp for the P2Y2-PCR product and two fragments of 408 bp 357 bp for the P2Y4-PCR product. The digestion products were also run into the gel.

Measurements of intracellular cAMP. cAMP was measured in ethanol extracts of cells grown on 24-well plates using a commercially available enzyme radioimmunoassay kit (Immunotech) which allows detection of cAMP in the range 0.5−50 nM with high reproductibility (accuracy ≥90%). This kit was described by the manufacturer as not reacting (cross-reactivity ≈10^4) with the other adenine nucleosides (AMP, cGMP or ATP). Results of cAMP determinations were expressed as picomoles/million cells. Extractions were carried out either after 3 min contact with increasing concentrations of ATP (10^{-6} M to 10^{-4} M) or at increasing times (0 to 7 min) after the addition of 10^{-4} M of ATP which was shown to be the concentration giving the maximal secretory response. 3-min contacts with agents were also carried out in order to determine whether they induce generation of cAMP: UTP, 2-MeSATP and ATP plus suramin (Bayer AG).

Single cell $[Ca^{2+}]_i$ measurements. MM39 cells were seeded at 10^4 cells/cm^2 onto glass coverslips and cultured for 48 h in complete culture medium. After four 1-h washes in serum-free medium, cells were incubated for 30 min in darkness at 37°C in a 10 mM Tris/HCl buffer containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1 μM Fluo 3/AM (from a 0.1 mM, 3% Pluronic F127 stock solution prepared in 20% dimethylsulfoxide). Changes in fluorescence were monitored at 37°C using the same incubation buffer and a fluorescence microscopy imaging system consisting of an Olympus IMT2 inverted phase contrast microscope with a ×40 objective lens and equipped for epifluorescence and photometry with a Lhesa 4015 SIT video camera as described in detail elsewhere [24]. When the baseline level of fluorescence had stabilized, 100 μM of ATP and UTP were added to the cell preparation.

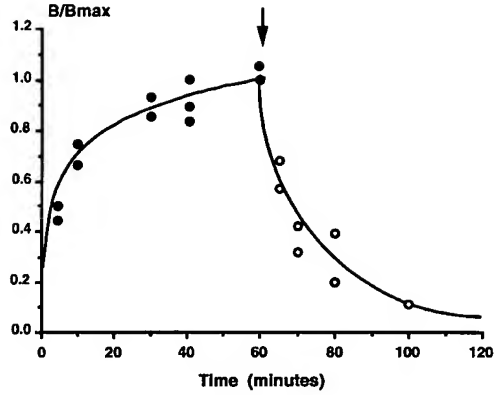

Fig. 1. Time-course for association (●) and dissociation (○) of [^{35}S]ATP[γS] binding to MM39 cells. Cells (10^6 cells) were incubated at 4 °C with [^{35}S]ATP[γS] and binding radiolabelling was sequentially counted. The dissociation was initiated by addition of 2.5 mM ATP to the cells (arrow) and bound radioactivity was measured for various periods of time. The figure shows a representative experiment. Data are expressed as the ratio of bound [^{35}S]ATP[γS] (B) to maximal [^{35}S]ATP[γS] bound (B_{max}).

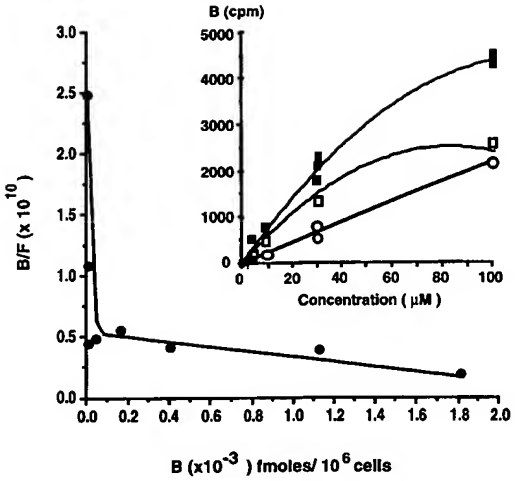

Fig. 2. Scatchard plot analysis of the dose dependence of [^{35}S]ATP[γS] binding to MM39 cells. Cells (10^6 cells) were incubated with increasing concentrations (0.1 μM to 100 μM) of [^{35}S]ATP[γS]. Non-specific binding (○) represents radioactivity in the presence of 2.5 mM ATP. Specific binding (□) refers to (total radioacivity bound; ■) − (non-specific radioactivity bound). Scatchard plot reveals a fit consistent with a two-site model. The high-affinity sites have K_d1 values of 2.5 ± 0.2 μM and the second low-affinity class sites has K_d2 values of 20 ± 5 μM).

Pharmacological stimulation of SLPI secretion. Confluent cultures of MM39 cells grown on 24-well plates were rinsed four times for 1 h with serum-free culture medium and then exposed for 30 min to 100 μM of nucleosides. 40 μl of the culture medium were harvested and the SLPI was directly measured by ELISA [25]. The SLPI secretory rate was defined as the ratio of the SLPI secreted in the assays to that secreted in control experiments which were plate wells in which only vehicle solutions were added at the same times as the wells where the drugs were added. Vehicle additions were shown to be ineffective on SLPI secretion by MM39 cells. In each experiment the mean SLPI secretory rate was determined from quadruplicate assays.

Statistics. All results were expressed as means ± SD made in quatriplicate. The significance between the effects of the concentrations of agents or between the effects of the agonists was determined by analysis of variance (ANOVA). The difference between the agents or between the concentrations of agents was isolated by the Scheffé's multiple comparison tests.

RESULTS

Binding kinetics of [^{35}S]ATP[γS] by MM39 cells. All experiments were performed at 4 °C in order to prevent hydrolysis of [^{35}S]ATP[γS] by ectonucleotidases.

Fig. 1 shows the association and dissociation of [^{35}S]ATP[γS] to MM39 cells. Association of [^{35}S]ATP[γS] was rapid (steady state was achieved within 30 min at 4 °C), stable and reversible. After the addition of an excess of ATP (2.5 mM), we can see that it displaced 90% of the [^{35}S]ATP[γS] bound on the cells over 40 min.

The data for the saturation binding of [^{35}S]ATP[γS] is shown in Fig. 2. Non-specific binding was linearly related to the radioligand concentration. It represented 25% of the total binding component at 10 μM [^{35}S]ATP[γS]. Scatchard analysis of the saturation curves obtained in four independent experiments showed that [^{35}S]ATP[γS] bound to two classes of non-interacting binding sites with high (K_d1 = 2.5 ± 0.2 μM) and low affinities (K_d2 = 20 ± 5 μM). Corresponding B_{max} values were 35 pmol/10^6 cells and 400 pmol/10^6 cells, respectively, an 11-fold difference in the number of binding sites per cell.

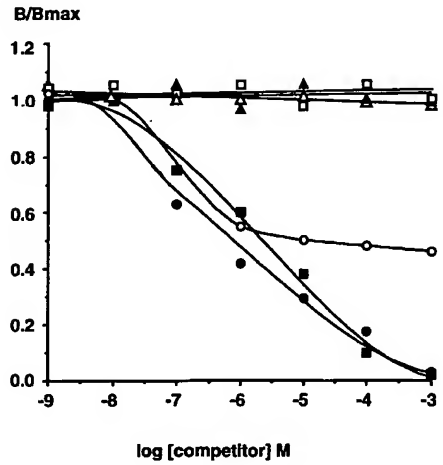

Fig. 3. Competitive displacement of 0.1 μM [^{35}S]ATP[γ^{35}S] by different nucleotides. (A) Competitive binding curves for increasing concentrations of adenosine (△) AMP (▲), UTP (□), ATP (●), ADP (○) and 2-MeSATP (■). (B) The corresponding logit transformation of the binding curves for ATP, ADP and 2-MeSATP give IC_{50} values of 0.5 μM, 2.3 μM and 19 μM, respectively. The data are representative of results from three separate experiments.

The binding specificity of [^{35}S]ATP[γS] was assessed by comparing the ability of various nucleotidic analogues to inhibit [^{35}S]ATP[γS] binding. Fig. 3 shows that ATP, ADP and 2-MeS-ATP prevented [^{35}S]ATP[γS] binding to MM39 cells in a concentration-dependent manner; UTP, AMP and adenosine up to 1 mM did not. Inhibition by ADP of [^{35}S]ATP[γS] binding was only partial. The IC_{50} values for concentrations of ADP needed to inhibit [^{35}S]ATP[γS] binding was 2.3 μM. In contrast, ATP and 2-MeSATP completely prevented [^{35}S]ATP[γS] binding, but the inhibition curves extended over more than two orders of

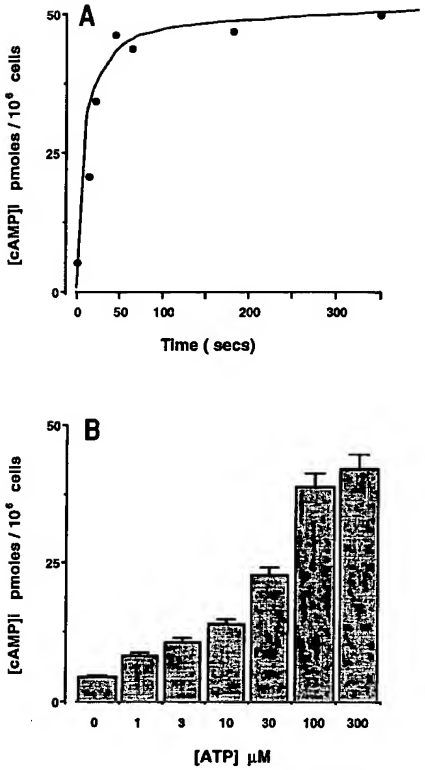

Fig. 4. Intracellular elevations of cAMP in ATP-stimulated MM39 cells. (A) Cells (10^6 cells) were stimulated with 100 µM ATP and sequentially lysed over time. Baseline $[cAMP]_i$ values was 4 pmol/10^6 cells. (B) Cells (10^6 cells) were stimulated with increasing concentrations of ATP and lysed after 3 min contact with the nucleotide. Curves are representative of data obtained from three independent experiments.

magnitude of competitor concentration. This suggested that ADP recognized a simple receptor site, while ATP and 2-MeS-ATP recognized two receptor sites. This result is consistent with the Scatchard analysis.

cAMP measurements. We next examined changes in $[cAMP]_i$ levels induced by ATP. Stimulation of MM39 cells with 10^{-4} M of ATP induced $[cAMP]_i$ formation (Fig. 4A). This increase was dependent on ATP concentration (Fig. 4B). Maximum values of $[cAMP]_i$ obtained were 48 ± 2.9 pmol/10^6 cells, a value 10 times higher than in unstimulated cells. Concentration of ATP which gives half of the maximal $[cAMP]_i$ increase was 18 µM.

We then investigated the ability of ATP analogues to induce elevations of $[cAMP]_i$. Neither UTP, nor 2-MeSATP used at submaximal concentrations (100 µM) were able to generate any elevation of $[cAMP]_i$ in MM39 cells (Fig. 5). Only ATP was able to induce elevations in $[cAMP]_i$ which was inhibited when ATP was added to the cells with 50 µg/ml of the P2-antagonist suramin.

Effects of UTP on MM39 cells. We next looked for the effects of UTP on biological responses by MM39 cells. Fig. 6A shows the action of nucleosides on SLPI secretion. Only ATP and UTP induced significant stimulation of SLPI secretion and to a similar extent ($+250 \pm 30\%$ and $+230 \pm 40\%$, respectively, compared with control experiment). No significant stimulation was observed with ADP, 2-MeSATP, AMP or adenosine. We also performed single cell $[Ca^{2+}]_i$ measurements which provide a

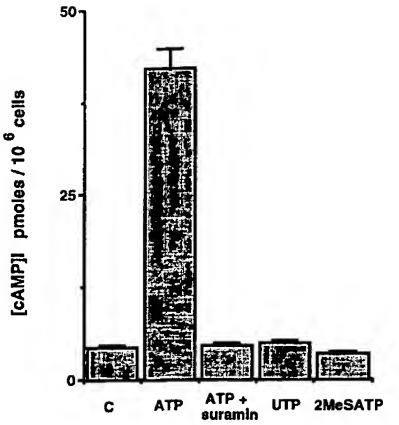

Fig. 5. Intracellular elevations of cAMP in human tracheal gland cells stimulated by different nucleotides. MM39 cells (10^6 cells) were stimulated for 3 min with 100 µM of ATP, UTP or 2-MeSATP, or 100 µM of ATP plus 50 µg/ml of suramin. The figure represents means ± SD for three independent experiments performed in duplicates.

convenient method for analysing agonist-mediated changes in $[Ca^{2+}]_i$. After the addition of 100 µM ATP or UTP, $[Ca^{2+}]_i$ rose rapidly and then declined (Fig. 6B). The maximum $[Ca^{2+}]_i$ level reached in response to ATP or UTP were 710 ± 80 nM ($n = 14$) and 760 ± 130 nM ($n = 7$), respectively, over a baseline $[Ca^{2+}]_i$, in unstimulated MM39 cells being 120 ± 40 nM.

RT-PCR analysis of the P2Y2 and the P2Y4 receptors. We checked for the presence of the P2Y2 and P2Y4 nucleotidic receptors by PCR amplification. Electrophoresis of the PCR products revealed that MM39 cells express both the P2Y2 and the P2Y4 receptor mRNAs (Fig. 7). To verify specificity of the PCR products, digestion with the restriction enzyme *PstI* was performed. The resulting digestion products of the amplified cDNAs migrated in the gel at the expected sizes (Fig. 7).

DISCUSSION

Tracheal glands serous cells express CFTR at high levels in the human bronchus, and represent an important target for the therapy of cystic fibrosis. Thus, it is relevant to clearly analyse and characterize the effects of therapeutic agents such as ATP and UTP (acting through P2 receptors) on these cells. We had previously given evidence for a P2u receptor on human tracheal gland serous cells based on the effects of nucleotide agonist action on intracellular calcium mobilization and on stimulation of protein secretion [18]. However in this previous work, we observed ATP-stimulated protein secretion at concentrations where intracellular calcium had not yet been mobilized. Faced with this intriguing result, the present study further characterizes the ATP-purinoceptor by its binding to [^{35}S]ATP[γS] analyses its coupling to another second-messenger system, the adenylyl cyclase pathway.

The activation of the P2u purinoceptor is described as being linked to increase in intracellular calcium through phospholipase C. It was also shown to be linked to inhibition of adenylyl cyclase [26] but, in some cases, also to generation of intracellular cyclicAMP ($[cAMP]_i$) in several cell types [27, 28]. Therefore, we examined the ability of ATP to induce changes in $[cAMP]_i$ levels. Extracellular ATP provoked a rapid increase in $[cAMP]_i$ by MM39 cells, which was characterized by a regular concentration/response relationship, and suggesting that the purinoceptor

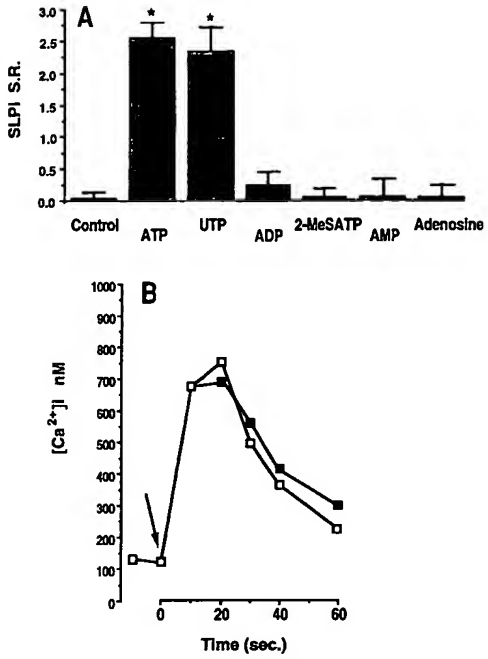

Fig. 6. Effects of UTP and ATP on MM39 cell secretion and $[Ca^{2+}]_i$ mobilization. (A) After four 1-h washes, MM39 cells were incubated for 30 min with 100 μM ATP, UTP, 2-MeSATP or AMP. SLPI secretory rate (SR) was determined as described in Materials and Methods. Only ATP and UTP stimulated equipotently SLPI secretion. (B) $[Ca^{2+}]_i$ mobilization was measured in single cells loaded with Fluo3/AM. ATP (□) and UTP (■) were used at 100 μM. Arrow, secretagogue addition to the cells. Data are from representative experiments.

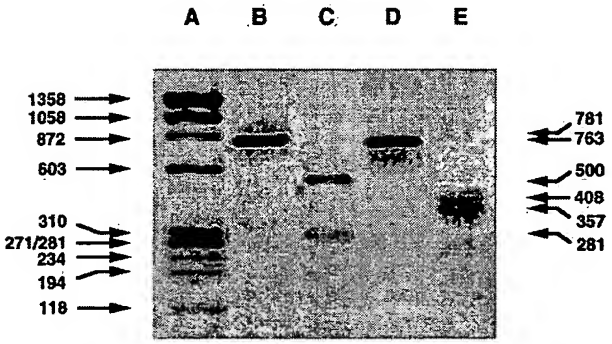

Fig. 7. RT-PCR detection of the P2Y2 and P2Y4 receptors in MM39 cells. After cDNA synthesis, samples were subjected to a 35-cycle PCR amplification. Amplification products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Lane A, molecular-size marker ΦX174/*Hae*III. Lanes B and D, P2Y2 and P2Y4 PCR products, respectively. Lane C and E, *Pst*I digestions of the P2Y2 and P2Y4 PCR products, respectively.

is positively coupled to adenylyl cyclase. Moreover, that adenosine is not a secretagogue of MM39 cells, suggesting an absence of the P1 receptor on these cells, and that the ATP-induced $[cAMP]_i$ generation is blocked by the P2-purinoceptor antagonist suramin strongly suggest that the ATP-induced generation of $[cAMP]_i$ is caused by stimulation of the P2-purinoceptors. This apparent coupling of the P2u receptor to adenylyl cyclase is in accordance with several other reports also describing a generation of $[cAMP]_i$ after extracellular challenge of ATP on epididymal cells [29], C2C12 myotubes [30] and bovine aortic smooth muscle cells [28]. The interaction of ATP with MM39 cells has three distinguishable effects, namely stimulation of protein secretion and mobilization of $[Ca^{2+}]_i$ and activation of adenylyl cyclase. A major question currently unresolved is whether these effects of ATP are mediated by a single type of receptor separately coupled to different pathways, or whether there are different binding sites coupled to different second messenger systems. The binding of [^{35}S]ATP[γS] to the receptor did not follow simple action mass kinetics. Scatchard plots of the equilibrum saturation binding measurements were curvilinear with a Hill coefficient of less than 1, and a two-site model best fit the data. K_ds observed from either Scatchard analysis or from association and dissociation rates constants were in the micromolar range and these K_ds as well as the presence of two [^{35}S]ATP[γS] binding sites were similar to those obtained in other cell systems [30, 31]. The fact that both K_d1 (2.5 μM) and K_d2 (22 μM) have values in the same magnitude order with the previous [18], reported EC_{50} values for 1) protein secretion (1 μM) and 2) $[Ca^{2+}]_i$ mobilization (20 μM) and $[cAMP]_i$ elevation (18 μM), is somewhat intriguing. It has been suggested that a rapid transition of the receptor to a desensitizated state with lower affinity to ATP may occur in PC12 cells [32]. Rapid desensitization of ATP-induced cellular responses have been reported [33, 34]. It has also been proposed that ATP may stimulate different second messenger systems through distinct purinoceptors [35]. One can suggest that the high-affinity receptor may trigger secretion and that when desensitized it induced $[Ca^{2+}]_i$ mobilization and$[cAMP]_i$ elevation in order to modulate magnitude of secretion. Although speculative, this model would explain why ATP can induce protein secretion at concentrations where no $[Ca^{2+}]_i$ mobilization and $[cAMP]_i$ elevation are yet generated (10^{-7} to 10^{-6} M) and also why this protein secretion stays maximal as soon as elevations of $[cAMP]_i$ and $[Ca^{2+}]_i$ occur (10^{-6} to 10^{-4} M). However, this obviously requires further support from studies specifically designed to test this hypothesis.

Our data also argue in favour of the presence on MM39 cells of a pyrimidoceptor specific for UTP. The P2u nucleotidic receptor has been characterized as a common receptor for ATP and UTP based on identical pharmacological effects [2], coupling to G-protein activation, and cross-desensitization by ATP and UTP [36]. However, existence of a pyrimidoceptor was suggested several times [37]. With the cloning and expression of the P2Y4 subtype [7, 8], the evidence that UTP is able to interact with a UTP-specific receptor located within the cell membrane now appears unquestionable. We found that in MM39 cells, ATP and UTP effects appear to be mediated by separate and distinct receptors. This conclusion is based (a) on the presence shown by RT-PCR amplification of the mRNA of the P2Y4 subtypes, (b) on the complete lack of UTP to compete with [^{35}S]ATP[γS] on the ATP-receptor, (c) on the finding that in contrast to ATP, UTP did not activate adenylyl cyclase but (d) on the equipotency of ATP and UTP to stimulate SLPI secretion and to generate similar $[Ca^{2+}]_i$ mobilization. These results are in keeping with those of Kim et al. [31] who also showed that UTP bound poorly with ATP[γS]-binding sites on guinea pig tracheal epithelial cells where a P2u receptor has primarly been pharmacologically evidenced.

These results also suggest the presence of another atypical ATP-specific P2Y receptor in MM39 cells. It is likely that the P2Y2 receptor may be present since we observed the expression of the P2Y2 gene and a equipotency of UTP and ATP to induce secretion and to mobilize intracellular calcium. However, the finding that ATP and UTP did not compete for the ATP[γS]-binding sites and are differently coupled to adenylyl cyclase suggest the presence of another ATP-specific receptor in MM39

cells. It is unlikely that this receptor may be the P2Y1 one because of the absence of a functional response to 2-MeSATP by the cells, the competition of 2-MeSATP for the ATP[γS]-binding sites rather suggesting that it is a partial antagonist. No secretory responses to ADP are also likely to favour an absence of P2Y3 receptors. The P2Y5 and P2Y7 receptors were first reported to be ATP-specific purinoceptors [9, 10] but it is now clearly demonstrated that these receptors can no longer be considered as purinoceptors [11, 12]. Thus, only a combination of the P2Y4, the P2Y2 and a yet unknown ATP-specific P2Y receptor could fit with all our results.

In summary, the present study gives evidence of the presence of both the P2Y2 receptor and of the P2Y4 pyrimidoceptor in HTG cells, and suggests the presence of an ATP-specific purinoceptor which appears coupled with generation of intracellular cAMP. Scatchard analyses of the binding data consistently show statistically reliable fits to a two-site binding model in which the low-affinity and the high-affinity site for ATP[γS] differ by one order of magnitude. The low-affinity site appears to be coupled with the elevation of $[cAMP]_i$. This receptor may be specific to ATP but not for UTP. The respective physiological role of these evidenced nucleotide receptors in regulating secretion by tracheal gland cells still needs to be clearly determined.

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